Biophysical characterization of mutants of *Bacillus subtilis* lipase evolved for thermostability: Factors contributing to increased activity retention

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Received 29 September 2011; Revised 14 December 2011; Accepted 8 January 2012

DOI: 10.1002/pro.2031

Published online 20 January 2012 proteinscience.org

Abstract: Previously, Lipase A from *Bacillus subtilis* was subjected to *in vitro* directed evolution using iterative saturation mutagenesis, with randomization sites chosen on the basis of the highest B-factors available from the crystal structure of the wild-type (WT) enzyme. This provided mutants that, unlike WT enzyme, retained a large part of their activity after heating above 65°C and cooling down. Here, we subjected the three best mutants along with the WT enzyme to biophysical and biochemical characterization. Combining thermal inactivation profiles, circular dichroism, X-ray structure analyses and NMR experiments revealed that mutations of surface amino acid residues counteract the tendency of Lipase A to undergo precipitation under thermal stress. Reduced precipitation of the unfolding intermediates rather than increased conformational stability of the evolved mutants seems to be responsible for the activity retention.

Keywords: lipase; directed evolution; iterative saturation mutagenesis; thermal inactivation; aggregation

Introduction

Enzyme stability, which includes thermostability, oxidative stability and resistance to undesired aggregation and precipitation, is critical in industrial applications of biocatalysts, and in biotechnology all of these factors contribute to the “robustness” of a protein. Numerous approaches have been reported to improve thermal and oxidative stability using site-

specific mutagenesis guided by rational design, yet further progress is hindered by the lack of understanding of the factors involved.^{1–3} For instance, intermolecular interactions can lead to significant aggregation and subsequent precipitation, a phenomenon which is difficult to predict. Directed evolution^{4–9} offers an alternative method for improving the robustness of proteins in biotechnology,¹⁰ with error-prone polymerase chain reaction (epPCR) and/or DNA shuffling being the most popular gene mutagenesis techniques in this endeavor. In many cases this approach is superior to rational design, although at the expense of having to screen large mutant libraries, which is still considered to be the bottleneck of directed evolution in general.¹¹ The need for thermostable proteins is so important that thermostabilization studies continue to be reported.^{12–17}

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: EC FP6 Research Infrastructure Action program (project EU-NMR); Grant number: RII3-026145.

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Figure 1. Thermal inactivation profiles of Lipase A variants determined at various conditions (panels A–C). Wild-type enzyme is represented by black lines, variant IX by blue lines, variant X by green lines and variant XI by red lines. Panel D shows the concentration-dependence of the activity retention of LipA variant XI at different temperatures. Error bars represent standard errors of the mean.

Recently, we proposed a different method to evolve enzyme thermostability^{18,19} by iterative saturation mutagenesis (ISM) applied at specific amino acid positions in an enzyme. To guide the decision-making regarding the amino acid sites to be altered, the B-FIT method was developed, in which residues with high B-factors in the crystal structure of the target enzyme are considered appropriate mutation candidates. The rationale behind this approach is the higher rigidity of thermostable proteins compared to their mesophilic counterparts at ambient temperatures,³ and the belief that targeted mutagenesis at flexible regions as indicated by high B-factors could lead to rigidification and thus thermostabilization.

We applied the B-FIT approach to *Bacillus subtilis* Lipase A (LipA).^{18,19} LipA is a mesophilic protein, which has been well characterized.^{20,21} Its three-dimensional structure was determined using crystals obtained at either basic conditions^{22,23} or at acidic pH.²⁴ In the past, the Rao group described the use of directed evolution based on epPCR to enhance the thermostability of LipA significantly.^{25–27} As a result of several rounds of ISM at sites displaying the highest B-factors in one of the X-ray structures,²³ we obtained mutants that retained significant activity after treatment at temperatures above 65°C, whereas the wild-type (WT) lipase lost all activity. In this study, we report biophysical investi-

gations for the three best B-FIT-evolved LipA mutants along with WT enzyme, thereby shedding light on the origin of their enhanced activity retention upon thermal treatment. We characterized these mutants using thermal inactivation profiles, melting experiments monitored by circular dichroism (CD) and structural characterization by X-ray crystallography and nuclear magnetic resonance (NMR). We show that the point mutations introduced during the evolutionary process significantly reduce the undesired precipitation tendency of LipA unfolding intermediates upon heating. This reduction of precipitation enhances the proficiency of an enzyme as a catalyst in biotechnology, an aspect of protein engineering that has not received much attention thus far.

Results and Discussion

Thermal inactivation profiles

Thermal inactivation profiles describe the residual activity retained after heating a protein sample and subsequent cooling. Previously, using the B-FIT approach, LipA variants were found with significant activity retention (measured using *p*-nitrophenyl caprylate as a substrate) after heating over 65°C and cooling down to room temperature. Three of these variants are studied here in detail, namely: variant IX (K112D, M134D, Y139C, I157M) obtained

in the 4th ISM step and used as parent for the last round of saturation mutagenesis, variant X (R33Q, D34N, K35D, K112D, M134D, Y139C, I157M) obtained in the last round of ISM, and variant XI (R33G, K112D, M134D, Y139C, I157M) also identified in the final ISM round.^{18,19}

Thermal inactivation experiments at neutral pH [Fig. 1(A)] reflect the selection conditions applied during the directed evolution process.^{18,19} As anticipated, WT protein retained almost no activity after heating and cooling, whereas the highest residual activity was found for variants X and XI, and slightly lower activity for variant IX. This high activity retention might originate from either improved thermodynamic stability, or from improved kinetic stability. Kinetic stability might be caused by, for example, more favorable refolding properties, reduced irreversible aggregation and/or precipitation of the mutants.

WT LipA does not retain its activity upon thermal treatment even at low concentrations (1 μ M) at neutral pH. However, when protein aggregation is limited, for example, at low pH²⁸ [Fig. 1(B)] or in the presence of guanidinium hydrochloride²⁹ [Fig. 1(C)], all LipA variants along with WT retained high activity levels comparable to the untreated protein even after application of high temperature. Therefore it can be concluded that in the absence of protein aggregation the refolding properties of WT and variants are similar.

Larger activity loss was observed at higher concentrations of the enzyme [Fig. 1(D)], again suggesting a role of protein precipitation in the activity recovery of LipA evolved mutants. Importantly, precipitation obscures results obtained using techniques that require high protein concentrations (e.g., CD or NMR).

Interestingly, the activity retention of the evolved variants is larger from species present at 80°C than from intermediates present at 60°C. Additional experiments in which the enzyme was kept at these temperatures for different time periods support this observation (Supporting Information Fig. S3). Since at pH 10 reconstitution from the 60°C intermediates becomes as efficient as from the 80°C intermediates (Supporting Information Fig. S2A), the retention of functional mutants from 60°C intermediates is affected by electrostatic interactions.

In the B-FIT study of LipA only surface residues were addressed and the majority of the mutations involved charged residues (two of four mutations in variant IX, five of seven in variant XI and three of five in variant XI). Additional thermal inactivation profiles determined using various buffer compositions (Supporting Information Fig. S2) indicated a role of the ionic interactions. Therefore, an altered surface charge distribution might be responsible for the enhanced activity retention upon thermal treatment of the evolved mutants.

In general, folding properties of the variants are similar to those of WT lipase, which is suggested by thermal inactivation profiles recorded when aggregation is reduced. This conclusion is further supported by molecular dynamics simulations, where similar unfolding patterns of WT and variant X were found (Supporting Information Fig. S8). Therefore, we concluded that folding pattern is unlikely to be responsible for the differences in activity retention upon thermal treatment of LipA WT and mutants. This leaves an increase of the thermodynamic stability of the fold, reduction of irreversible aggregation propensity of the unfolding intermediates and insolubility as possible explanations of the efficient reconstitution of the evolved mutants upon thermal treatment.

Characterization of recovered of LipA variant XI by NMR spectroscopy

An important point concerns the question whether the enzyme recovers its tertiary structure after the thermal inactivation experiment. It was tested using NMR spectra recorded for native and thermally treated ¹⁵N-labeled variant XI LipA.

Heating and cooling down of diluted LipA variant XI solution produced a decent amount of protein for the NMR experiments. However, when WT enzyme was subjected to a similar procedure, no significant amount of soluble protein was obtained due to protein precipitation even from the diluted solution.

After heating and cooling variant XI shows a conformation very similar to the untreated protein, as peaks in 1D ¹H and 2D [¹⁵N,¹H]-HSQC spectra (Fig. 2) do not change their positions dramatically after thermal treatment. Despite the high similarity of the HSQC spectra, there are certain subtle differences between native LipA variant XI and protein treated at high temperature (see Supporting Information Fig. S1).

In addition, it was observed that not all protein recovers its native fold. Some small amount of soluble unfolded and/or degraded species can be seen in the spectra, although no significant change in the specific activity was found when comparing native and recovered LipA variant XI. This indicates that the fraction of unfolded/degraded and also irreversibly aggregated (but still soluble) species is small. Based on the experiments with preparation of thermally treated Lipase A WT and variant XI, it can be concluded that these irreversibly aggregated soluble species are not responsible for the activity loss upon thermal treatment.

In general, LipA variant XI recovers a conformation almost identical with the native protein according to the NMR data. This observation agrees with the activity recovery observed after thermal treatment.

Figure 2. NMR spectra recorded for native and thermally treated ^{15}N -labeled Lipase A mutant XI. (A) 1D ^1H spectra. (B–D) 2D ^{15}N , ^1H -HSQC spectra of mutant XI Lipase A: (B) Native. (C) Recovered after 60°C treatment. (D) Recovered after 80°C treatment.

Protein denaturation studies using CD

The thermodynamic stability of LipA was studied using far UV CD, which is an established tool to study protein unfolding and precipitation.³⁰ Contrary to the thermal inactivation profiles, the protein is directly studied during the actual melting process. Examples of denaturation experiments performed for LipA variants at pH 7 are shown in the Supporting Information (Fig. S4). In this study the following parameters were monitored:

1. The “unfolding initiation temperature” (T_i), which is defined as the temperature at which the unfolding transition begins. Technically, T_i is determined as the temperature where the first melting slope intercepts the ellipticity value of the native protein recorded before protein melting. T_i is used instead of melting temperature (a temperature at the midpoint of the unfolding transition) due to the fact that many melting curves indicate multiple melting transitions.
2. High-tension voltage jumps, which are an indication of solution turbidity and therefore reflect protein precipitation.³¹
3. The CD melting slope value of the first unfolding transition, which indicates how fast the CD signal at 222 nm is being lost. This quantity relates to the rate of protein unfolding and/or precipitation.

Since in the previous section we showed that the LipA melting behavior depends strongly on the pH of the solution, we collected CD data at various pHs. Certain trends related to variant thermodynamic stability were observed (Fig. 3). The T_i values indicate that WT enzyme is actually slightly more stable than the mutants, as the T_i values for WT are a few degrees higher than the corresponding values for the mutants across the studied pH range [Fig. 3(A)]. Similar observations were made in the

presence of various additives (salt, chaotropes, see Supporting Information Table S1 for details).

For most pH values, the slope of ellipticity versus temperature is steeper for WT LipA [Fig. 3(B)] than for the variants. This indicates that although the variants start unfolding at lower temperature than WT, the loss of signal as a function of temperature proceeds slower than in the case of WT. Thus, early unfolding intermediates formed at the beginning of the melting process of WT seem to be more prone to signal loss than the early intermediates formed by the mutants.

The pH range in which no protein precipitation occurs differs slightly among the variants [Fig. 3(C)] and roughly corresponds to theoretical pI values. This suggests an important role of ionic interactions in the LipA precipitation and the differences can be explained by the fact that mutations at sites 33–35, 112, and 134 involve charge alterations. Since these residues are located on the protein surface, they might influence intermolecular interactions leading to protein aggregation and precipitation upon thermal stress.

Steeper slopes of ellipticity upon temperature increase are observed in the pH range where the protein precipitates upon heating, which suggests that precipitation contributes to a large extent to the signal loss upon protein heating. It seems that the variants evolved in our laboratory exhibit higher kinetic stability³³ than WT enzyme, since the intermediates formed by the mutants at elevated temperature precipitate slower than intermediates formed by LipA WT.

Activity retention upon thermal treatment of LipA variants does not originate from the enhanced thermodynamic stability of the lipase fold, as the mutants start to melt at lower temperature than WT. The kinetic stability was achieved at the expense of thermodynamic stability. This is a

Figure 3. Melting of Lipase A variants at various pH values observed by CD. Black diamonds connected by solid black lines represent values determined for WT protein, dark gray-filled squares connected by dashed black lines correspond to variant IX, empty triangles connected by dotted black lines correspond to variant X and light gray-filled circles connected by solid gray lines correspond to variant XI. (A) Unfolding initiation temperatures, T_i . (B) Slopes of the first unfolding transition. (C) pH ranges where no precipitation was observed are covered by black lines. Predicted pI values³² are indicated as gray dots.

surprising observation considering a previous account on LipA evolution towards thermostability. Using error-prone PCR as the randomization method, the Rao lab obtained LipA variants that exhibit significantly higher thermodynamic stability than WT. Interestingly, these mutants show higher kinetic stability as well, since they unfold reversibly upon thermal treatment. All of the mutations introduced in that study seem at the protein surface,^{25–27} similarly to our case.

The data indicates that a lower precipitation propensity of unfolding intermediates of variants compared to WT is responsible for the activity retention of the mutants evolved using B-FIT. Neither

significant changes of refolding properties (based on thermal inactivation profiles), formation of soluble irreversibly aggregated species, nor increased thermodynamic stability (CD data) of the mutant proteins explain our results.

The evolutionary pressure targeted activity retention, which was used as a screening method, and B-FIT succeeded in evolving mutants retaining their activity upon thermal treatment. However, the fold stability was not screened for, and therefore the creation of more thermodynamically stable mutants, although possible, has not occurred.

Crystal structure of LipA variant X

Efficient renaturation of the evolved LipA mutants upon thermal treatment must originate from properties at the molecular level. The X-ray crystal structure of the LipA variant X was determined at 1.85 Å resolution. Refinement statistics and stereochemical quality of the final model (analyzed using the MolProbity webserver) are listed in Table I.

Table I. Data Collection, Structure Determination, and Refinement Statistics of LipA Mutant X

X-ray data collection	
Wavelength (Å)	0.8726
Space group	$P2_1$
Cell dimensions	$a = 59.2$ Å, $b = 45.5$ Å, $c = 77.6$ Å
	$\alpha = 90^\circ$, $\beta = 101.1^\circ$, $\gamma = 90^\circ$
Resolution	45.5–1.85 Å (1.95–1.85 Å)
R_{merge}	0.094 (0.333)
Number of measured reflections	82119 (11934)
Number of unique reflections	33941 (5011)
$I/\sigma(I)$	8.8 (3.3)
Completeness (%)	97.4 (98.9)
Crystallographic refinement	
Asymmetric unit content (two monomers)	
Number of (nonhydrogen) protein atoms	2720
Number of water molecules	482
Other ligands	Two chloride ions, two sulfate ions, five glycerols
Average B-factor (Å ²) (protein)	9.3 (chain A)/13.0 (chain B)
Average B-factor (Å ²) (waters)	26.7
Wilson B (Å ²)	13.3
$R_{\text{cryst}}/R_{\text{free}}$	0.175/0.223
Stereochemical quality of the model	
RMSD bond lengths (Å)	0.011
RMSD bond angles (°)	1.11
Ramachandran plot (favored)	96.4%
Ramachandran plot (additionally allowed)	3.6%
Ramachandran plot (outliers)	0.0%

Numbers in parentheses represent the parameters for the highest resolution shell.

Figure 4. (A) RMSD from mean structure coordinates determined for each residue of LipA variant X and WT represented across the amino acid sequence. Gray boxes indicate mutation sites. (B) Normalized B-factor differences determined for LipA variant X and WT residues presented along the amino acid sequence. Error bars in panels A and B represent standard errors of the mean. (C) RMSD of mean structures shown in panel A presented using the LipA variant X structure. The color gradient from yellow to blue indicates increasing RMSD values. Balls and sticks represent side chains introduced during directed evolution. The location of the active site is indicated by a large violet ball. (D) Normalized B-factor difference illustrated in panel B represented using the structure of variant X. The representation is analogous to panel C using rainbow colors corresponding to the normalized B-factor difference. An interactive view is available in the electronic version of the article.

The structure of variant X was compared with all WT LipA entries in the PDB (1I6W, crystallized at pH 10.0²²; 1ISP, pH 8.7²³; 2QXT, pH 4.5²⁴; 2QXU, pH 5.0²⁴). Figure 4(A) shows the coordinate RMSD between structures of the LipA variant X and WT reported in the literature. The backbone RMSD is small for all residues; side chain conformations are exclusively responsible for the large RMSD values found for certain residues.

Overall, the core of variant X is very similar to the structures of the WT protein, as expected for an active protein. However, several residues on the surface of the protein have different side chain conformations [Fig. 4(C)]. They are either involved in intermolecular contacts in the crystal lattice of variant X (F17, W42, Y49, N120, M137, R142, Y161) or are interacting with such residues (K23, K44, P119, Q121, K122, N181). Moreover, five out of seven

residues (Q33, N34, D35, C139, I157), which were introduced during directed evolution, make new intermolecular contacts, which results in a completely different packing of variant X in the crystals. Figure 5 illustrates the intermolecular contact surfaces where the introduced mutations induced structural differences.

The B-FIT method was designed to address protein sites with the highest B-factors with the aim of obtaining more rigid proteins by applying evolutionary pressure on these sites. As a result, the mutated residues at these sites would be expected to have lower B-factors. We compared the B-factors of variant X against WT depositions from the PDB; the differences in the normalized B-factors across the sequence (see Materials for details) are presented in Figure 4(B). Contrary to the coordinate RMSD values, B-factors of backbone and side chains follow similar trends.

Figure 5. Intermolecular interactions of residues with altered side chain conformations of variant X compared to WT LipA. Residues of interest are presented as thick lines. The protein main chain is represented by thin lines, where one of the interacting monomers is represented in green with residue labels in blue, and the other one in pink lines and black labels. Relevant hydrogen bonds are represented by dotted black lines. An interactive view is available in the electronic version of the article.

No significant B-factor differences were observed in the protein core; however, differences do occur in several loop regions [Fig. 4(D)]. One region, between residues 14 and 21, displays lower B-factors, but higher B-factors were found in the regions 60–70, 129–153, and 177–181 (C-terminus). On average, variant X has higher B-factors than WT lipase A. These higher B-factors suggest decreased protein rigidity, which is in agreement with the lower thermodynamic stability of the mutants as determined by CD melting experiments.

In addition, none of the mutation sites exhibits significantly lower B-factors, and residues 134 and 139 were even found to have higher B-factors. Although these findings may suggest that the B-FIT approach is flawed, it should be realized that the selection of mutants was based on retention of activity,^{18,19} which is not the same as thermodynamic stability. Therefore, B-factors do not have to be decreased and protein rigidity does not have to be improved along with improving activity retention. Indeed, our system found another outlet for the evolutionary pressure (i.e., reduced precipitation under thermal stress) than increase of protein rigidity.

Generally, directed evolution of enzymes was shown to be capable of increasing thermodynamic

stability before. This can be well rationalized, for example, by reducing van der Waals strain as was found for TEM-1 β -lactamase,³⁴ substitution of hydrophobic residues on the surface, increase of secondary structure propensity or removal of flexible side-chains.¹³ Often both thermodynamic and kinetic stability of an enzyme are improved by *in vitro* evolution simultaneously. It happened to the LipA in the previous study, where loop tightening aided by hydrogen bonding,²⁷ electrostatic repulsion and limited conformational freedom around proline were indicated as factors stabilizing the enzyme.³⁵ Another example relates to hydrophobic packing or tethering the terminus to the protein core in adenylate kinase.³⁶

It is a common practice that the literature reports do not establish whether the evolved thermostable biocatalyst is thermodynamically or kinetically stable, or both. Large fractions of the thermostability assays used in directed evolution are based on activity retention following heat treatment (T_{50} values); which might result from either thermodynamic or kinetic stability. Whereas thermodynamic stability can be explained at the molecular level, there are no simple structural elements pinpointed to the improved kinetic stability of the enzyme so

far.³⁷ Our efforts have not produced any clear evidence in this field as well.

Conclusions

We have previously improved the retention of activity after thermal treatment of the Lipase A from *Bacillus subtilis*.^{18,19} In this study, we have characterized the evolved mutants by a variety of biophysical investigations, which allow a closer look on the evolutionary process leading to enhanced activity retention upon thermal treatment of LipA.

We found that the evolutionary process resulted in a decreased tendency for precipitation of unfolding intermediates of the mutants, an undesired process that occurs when heating WT LipA. In fact this is likely the explanation why the evolved LipA mutants are able to retain their activity upon treatment. These mutants turned out to be more stable kinetically instead of thermodynamically. We also found that the mutations alter the surface properties of the protein at the expense of a slight decrease of the thermodynamic stability of the protein. In addition, the evolutionary process changed some of the catalytic properties of the enzyme (Supporting Information Figs. S5 and S6).

Although the B-FIT method was originally developed to target thermostability of proteins, current data shows that, at least for LipA, other factors also seem to guide the outcome of the procedure. First of all, the screening method is a crucial component of the experimental setup during directed evolution, as it determines the results. Increased rigidity of the protein fold does not necessarily have to be obtained when screening targets activity retention. Furthermore, reduced precipitation may sometimes be another outlet for the evolutionary pressure and therefore obscure the outcome of the B-FIT process, as we report here for LipA.

To conclude, both the thermodynamic stability and aggregation propensity of native proteins (i.e., solubility) have been subjects of directed evolution studies before.^{9,38} However, the irreversible aggregation and precipitation propensity of the thermally unfolded states has not been commonly addressed by directed evolution so far. In our study, this phenomenon is the major reason for activity retention of the evolved mutants. Reducing precipitation of the thermally unfolded states can therefore be a novel approach for optimizing enzymes.

Methods

Construction of plasmids for lipase expression

WT LipA and mutants were cloned into pET-22b(+) (Novagen) plasmid before directed evolution studies.¹⁸ To obtain expression of lipase without any tag, the *pelB* leader sequence was removed. The following primers (obtained from Invitrogen) were used for PCR:

- 5'-CTTTAAGAAGGAGATATACATATGGCTGAA CACAATCCAGTCGTTATG-3' and antisense;
- 5'-CATAACGACTGGATTGTGTTTCAGCCATATG TATATCTCCTTCTTAAAG-3'.

PCR was run using KOD DNA Polymerase and a T Gradient thermocycler (Biometra). All the components except primers and matrix DNA were obtained from Novagen. Matrix DNA was then digested using DpnI endonuclease (New England Biolabs). The PCR product was used to transform *Escherichia coli* DH5 α chemically competent cells. After colony selection and culture growth, DNA was extracted and clone correctness was proven by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Protein preparation

Expression. After transformation and selection on LB agar plate with carbenicillin (100 mg/L in all growth media described here), *E. coli* BL21 (DE3) RP cells were grown in 1.25 mL of LB medium with carbenicillin at 37°C. An Infors HT Ecotron shaker was used throughout the expression procedure. The cells were harvested and resuspended in 25 mL of M9 medium with carbenicillin. The culture was grown overnight at 37°C, harvested and inoculated into 500 mL of M9 medium with carbenicillin. To prepare ¹⁵N-labeled mutant XI, 1.0 g of ¹⁵NH₄Cl (Cambridge Isotope Laboratories) per 1 L of M9 medium was used as a sole nitrogen source. Labeled M9 medium was used as the final expression broth, as well as the preculture medium. The culture was grown at 37°C. After having reached an OD₆₀₀ of 1.8–2.0 (typically after 3 to 4 h after inoculation), protein expression was induced using 0.75 mM isopropyl β -D-thiogalactopyranoside (Fermentas). Protein expression was carried out for 2.5 days, after which the cells were harvested and frozen at –20°C.

Protein extraction. The pellet was thawed and refrozen at –20°C. Then the frozen cells were resuspended in 40 mL of lysis buffer (10 mM sodium phosphate, pH 8.0, 50 mM NaCl, 1 mg/mL of lysozyme (AppliChem) and 10 U/mL of DNaseI (AppliChem)) and incubated at room temperature for 2 h. The lysate was centrifuged for 10 min. at 17,000g at room temperature. Lipase is present in both supernatant and pellet, and therefore both fractions were collected. The insoluble fraction was reconstituted by suspending the pellet in 5 mL of 6 M guanidinium hydrochloride and gradually diluting the sample to 100 mL of total volume using 10 mM sodium phosphate, pH 7.0. This solution was spun for 10 min. at 17,000g at room temperature and supernatant was collected. The pellet was subjected to a second round of protein

extraction. Supernatants with reconstituted lipase were kept at room temperature overnight. The mixture was centrifuged for 10 min at 17,000g at room temperature, the supernatant was collected and filtered through 0.22 μ m pore filters. The filtered solution (~250 mL) and the soluble fraction of the cell lysate (~40 mL) were subjected to the purification procedure separately.

Protein purification. An AKTA Purifier (GE Healthcare) was used for chromatography. Hydrophobic interaction chromatography was performed using three connected HiTrap Phenyl 5 mL (GE Healthcare) columns equilibrated with 10 mM sodium dihydrogen phosphate (pH 5.5) with 0.03% NaN₃ (equilibration buffer). The filtered supernatant was loaded onto the columns and washed using equilibration buffer. Lipase was eluted using 70% ethylene glycol in equilibration buffer. Fractions containing high lipase activity were subjected to cation exchange chromatography using a HiTrap SP HP 5 mL column (GE Healthcare) in equilibration buffer. Active fractions were loaded and the column was washed using equilibration buffer. Lipase was eluted using 20% elution buffer (10 mM sodium phosphate, pH 7.0, 500 mM NaCl, 0.03% NaN₃) mixed with 80% equilibration buffer. A relatively large amount of protein forms aggregates on the column. The aggregates were removed from the column using 6 M guanidinium hydrochloride, and refolded by 20-fold dilution in 10 mM sodium phosphate buffer, pH 7.0, 0.03% NaN₃. Both fractions were concentrated. Buffer exchange to 10 mM sodium phosphate, pH 7.0, 0.03% NaN₃ was performed using 10 kDa Amicon centrifugal filtration devices (Millipore). Finally, the protein was concentrated to ~10 mg/mL. Protein purity was assessed using SDS-PAGE and electrospray ionization mass spectrometry. Routinely, the procedure yielded ~20 mg of pure Lipase A from 0.5 L of culture.

Buffers used for characterization

The following buffers were used for the biophysical assays: 10 mM sodium acetate for measurements at pH 4.0–5.5; 10 mM sodium phosphate for studies at pH 6.0–8.0; 10 mM glycine-NaOH for studies at pH 9.0–10.5; 10 mM sodium bicarbonate, pH 11.0; 10 mM NaOH, pH 12.0; 10 mM sodium phosphate, pH 7.0, with 1 M NaCl; 10 mM sodium phosphate, pH 7.0, with 1 M guanidinium hydrochloride; 10 mM sodium phosphate, pH 7.0, with 2 M urea; 10 mM sodium phosphate, pH 7.0, with 4 M urea; 10 mM sodium phosphate, pH 7.0: ethylene glycol 1:1 (v/v). All buffers contained 0.03% sodium azide to prevent bacterial growth.

Thermal inactivation profiles

LipA (between 25 and 50 μ g/mL) was prepared in different buffers. Hundred microliters of the solution

was pipetted into a 96-well PCR plate and the plate was exposed to thermal treatment using a T Gradient thermocycler (Biometra). The program consisted of 5 min. warming at 37°C, followed by a 15 min. treatment at various temperatures ranging from 37 to 97°C, followed by 5 min at 4°C and finally 15 min at room temperature. The residual activity was measured using the following assay: 10–30 μ L of the heated lipase solution (combined with water to give 30 μ L of volume overall) was mixed with 100 μ L of assay buffer: 50 mM potassium phosphate, pH 8.0, 0.1% of Triton X-100, 5% (v/v) acetonitrile and 2.5 mM *p*-nitrophenyl caprylate. Formation of the reaction product (*p*-nitrophenol) was followed colorimetrically at 405 nm using Spectramax Plus plate reader (Molecular Devices) in 96-well plate format. The activity retained by samples kept at 37°C was used as a reference (100%).

Concentration dependence of LipA mutant activity loss

Various concentrations (1–15 μ M) were prepared of mutant XI in 10 mM sodium phosphate, pH 7.0, 0.03% sodium azide. Thermal treatment and residual activity determination were performed as described for thermal inactivation profile experiments, except that a constant temperature of 60 or 80°C was applied instead of a temperature gradient.

NMR experiments

LipA mutant spectra were measured in 10 mM sodium phosphate, pH 7.0, 5 mM D,L-1,4-dithiothreitol-*d*₁₀ (Cambridge Isotope Laboratories), 10% D₂O and 0.03% sodium azide. 1D ¹H- and 2D [¹⁵N,¹H]-HSQC NMR spectra were recorded at 298 K using a Bruker Avance 600 spectrometer equipped with a 5 mm CPTCI 1H-13C/15N/2H Z-GRD cryogenic probe.

Preheated ¹⁵N-labeled mutant XI samples were prepared as follows: native protein was diluted to 1 μ M using 10 mM sodium phosphate, pH 7.0, 0.03% sodium azide. Aliquots of 100 μ L were pipetted into a 96-well PCR plate and samples were incubated at 60 or 80°C for 15 min using a T Gradient thermocycler (Biometra). Then the combined aliquots were concentrated using 10 kDa Amicon centrifugal filtration devices (Millipore).

¹⁵N-labeled LipA mutant XI NMR sample concentrations: native protein: 0.5 mM, protein heated at 60°C: 0.1 mM, protein heated at 80°C: 0.3 mM.

CD measurements

CD spectra and melting curves were recorded using a Jasco J-810 spectropolarimeter. Two hundred microliters of 0.3 mg/mL Lipase A in the appropriate buffer was routinely used for measurement in quartz cuvettes of 1 mm optical length. Melting curves were recorded at 222 nm wavelength, using 1°C steps with a rate of 1°C/min. The curve was recorded

starting from 25 to 95°C. Afterwards, a reverse cooling curve was recorded using similar settings.

X-ray structure determination of LipA mutant X

Crystallization. Crystallization experiments were performed using the hanging drop vapor diffusion method at 293 K. Drops were prepared by mixing 1.25 μ L of protein solution (10.3 mg/mL LipA mutant X in 30 mM BES-NaOH buffer, pH 7.0, 0.03% (w/v) NaN₃) with 1.25 μ L of reservoir solution (24% (w/v) PEG 3350, 0.2 M (NH₄)₂SO₄, 0.1 M BisTris-HCl buffer, pH 6.0), and equilibrated over 1 mL of reservoir solution. Crystals seemed within 1 week and reached a typical size of about 800 $\times$ 100 $\times$ 20 μ m; they were cryo-protected using 28% (w/v) PEG 3350, 0.05 M (NH₄)₂SO₄, 0.05 M BisTris-HCl buffer, pH 6.0.

Data collection and structure determination. A single data set was collected at 100 K, at beam line ID23-2 (ESRF, Grenoble, France); diffraction images were integrated and scaled with XDS.³⁹ Data collection statistics are listed in Table I. The structure of LipA mutant X was solved by the molecular replacement method using Phaser⁴⁰ with the coordinates of WT *Bacillus subtilis* LipA²² (PDB code: 1I6W) as the search model.

Refinement and model building. Refinement of the LipA mutant X structure was performed at 1.85 Å resolution using REFMAC5⁴¹; cycles of restrained refinement (including twofold NCS) were alternated with manual model building in COOT.⁴² The final model comprises two monomers containing residues 2–181; only the N-terminal residue was not observed in the electron density. Each of the seven mutated residues is clearly visible in the electron density. The final R-factors are 0.175/0.223 ($R_{\text{cryst}}/R_{\text{free}}$). Structure analysis was performed using PyMOL (DeLano Scientific LLC) and MOLMOL.⁴³ Figures were prepared using PyMOL and VMD.⁴⁴ The coordinates and structure factor amplitudes have been deposited in the PDB (accession code 3QZU).

Comparison of B-factors between LipA WT and variant X. B-factors of the variant X were compared with all WT depositions from the PDB (1I6W,²² 1ISP,²³ 2QXT, and 2QXU²⁴). Because B-factors cannot be compared directly, scaling of the B-factors was necessary. As an internal reference for this purpose, we used the average B-factor of backbone atoms that were common to all analyzed chains excluding atoms involved in crystal contacts (within 6 Å distance from any atom belonging to another chain). Furthermore, B-factor outliers were excluded from the reference set as described by Smith *et al.*⁴⁵ Scaling by the reference yielded normalized B-factors (real B-factor divided by the reference value); these normalized B-factors were

then averaged over all analyzed chains for every atom separately. Averaged normalized B-factors obtained for WT were subtracted from those obtained for variant X for each atom individually. These differences were then averaged over all atoms of each residue.

Acknowledgments

W.A. was Marie Curie Outgoing International Fellow sponsored by the European Communities. The authors are grateful to the scientists of beam line ID23-2 (ESRF, Grenoble) for help during data collection. Financial support by the EC FP6 Research Infrastructure Action program (project EU-NMR; contract 26145) is gratefully acknowledged.

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